Autoregulation of *hip*, an Operon That Affects Lethality due to Inhibition of Peptidoglycan or DNA Synthesis

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The *hip* locus of *Escherichia coli* affects the frequency of persistence to the lethal consequences of selective inhibition of either DNA or peptidoglycan synthesis. Regulation of the *hip* operon, which consists of a regulatory region and two genes, *hipB* and *hipA*, was examined with strains containing a *hip-lac* transcriptional fusion placed in single copy at the λ *att* site. Disruption of the *hip* locus increased activity from the fusion 16-fold. Repression was restored by supplying HipB in *trans*. HipB was overexpressed and purified. On the basis of gel filtration and cross-linking studies, HipB is a dimer in solution. Sequence analysis revealed that HipB is a Cro-like DNA-binding protein. The interaction of HipB with the *hip* regulatory region was examined by gel retardation, DNase I protection, and methylation protection studies. HipB binds with a K_{app} (*K* apparent) of 40 pM to four operator sites with the conserved sequence TATCCN$_8$GGATA (N represents any nucleotide). Binding to the operators is nearly simultaneous and appears to be cooperative. Analysis of the role of HipA in the regulation of the *hip* operon is complicated by the toxicity of HipA in the absence of HipB. Strains disrupted in *hipB* but not in *hipA* could not be recovered. Moreover, *hipA*-containing plasmids cannot be replicated in strains defective in or lacking *hipB*. HipA is found exclusively in a tight complex with HipB. Although disruption of *hipA* slightly increased expression from the *hip-lac* fusion, in vitro studies suggest that HipA does not bind to the *hip* regulatory region directly but indirectly via HipB.

The vast majority of a population of bacteria are killed after prolonged inhibition of synthesis of either peptidoglycan (3) or DNA (30). However, a small but clinically significant fraction, 10^{-6} to 10^{-5}, survives, a phenomenon termed persistence by its discoverer (3). Genetic analysis of persistence in *Escherichia coli* was initiated by isolating high-frequency-persistence mutants (Hip) (4, 25). The frequencies of persistence of these mutants are 1,000- to 10,000-fold greater than those of the corresponding parental strains (4, 25, 30). The mutations were mapped to min 33.8 on the *E. coli* chromosome (25). This locus, designated *hip*, is in the terminus region (17) close to *terC3* (16). Nucleotide sequence analysis revealed that the *hip* operon contains a short open reading frame, *hipB*, followed by a second, longer open reading frame, *hipA* (4). Mutations in either *hipB* or *hipA* result in the Hip phenotype (4, 25). These mutations are believed to be point mutations because they were created with ethyl methanesulfonate and are readily revertible.

In addition to the two open reading frames, the *hip* operon contains what appears to be an intricate regulatory region (4). The 5′ noncoding region of *hip* contains the −35 and −10 promoter elements as well as a number of inverted repeat sequences (Fig. 1). There are three similar inverted repeats with the conserved sequence TATCCN$_8$GGATA (N represents any nucleotide) as well as a longer inverted repeat upstream of the *Eco*RV restriction site. Also present in the 5′ noncoding region is a sequence nearly identical to the integration host factor (IHF) consensus binding site 5′-(A/T)ATC AAN$_4$TT(A/G)-3′ (11) (Fig. 1).

Preliminary observations made with *hip-galK* fusions in multicopy plasmids suggested that HipB represses the *hip*

promoter (4). In this report, a more definitive analysis of *hip* promoter regulation is conducted with single-copy promoter fusions. In addition, the interactions of the *hip* gene products with the *hip* regulatory region are examined in vitro with purified protein preparations.

MATERIALS AND METHODS

Reagents. Unless otherwise specified, chemicals were purchased from Sigma. Radiochemicals were from DuPont/NEN. Restriction enzymes were from Boehringer-Mannheim or New England Biolabs. DNase I, calf intestinal alkaline phosphatase, and bovine serum albumin (BSA) were from Boehringer-Mannheim. T4 polynucleotide kinase and sonicated salmon sperm DNA were from Pharmacia. 3,3′-dithiobis(sulfosuccin-imidyl propionate) was purchased from Pierce. Gel electrophoresis chemicals were obtained from Bio-Rad.

Bacteria, plasmids, and phages. The bacterial strains, phages, and plasmids used in this study are described in Table 1. Deletions and insertions in the *hip* locus were created by a modified version of the method of Gutterson and Koshland (15).

Δ*hipBA*::*tetA*. The *hip* promoter, *hipB*, and part of *hipA* were deleted from the chromosome by replacing the 788-bp *Eco*RV fragment of *hip* (4) with *tetA* of pBR322. The *polA* strain, HM2005, was transformed with pNO2295tet. Plasmid integrates, Amp^r Tet^r, were grown for 10 generations in LB broth (24), and Amp^s Tet^r colonies were chosen for further analysis. The putative *hip* deletion, Δ*hipBA*::*tetA*, was transferred from HM2006 to the desired backgrounds by P1 transduction. Verification of the deletion in these strains was made by Southern analysis (33) with the radiolabeled 788-bp *Eco*RV *hip* fragment as the probe.

***hipA*::*cat*.** *hipA* was interrupted by inserting a 952-bp fragment containing the Cm^r determinant, *cat*, at the *Hpa*I restriction site located 47 bp downstream of the translational start of

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Sna BI

AGTACGTACGGTGAGGCGAAAGTAAAATAACAAGCCTGTGGGGAATGTCGTGTGCAACTAGGTGATAAAGGCTATAGCGATACTCAGGGCATGCTGG

<center>•   •   •   •   •
-280   -260   -240   -220   -200</center>

<center>IHF</center>

GAGTGAAATATAGCTGGTAAACCGTA**TAAGCCGCATG**TCGAGATGG**CATGCGGCTTA**ATATAGCCGACTTCAAACGCGCATCAACGCCTTATTTAGA

<center>•   •   •   •   •
-180   -160   -140   -120</center>

Eco RV   -35   -10

TCCTCCTTTGTTACCGATCGCGGATATCGCAGCGT**TTATCCC**GTAGAGC**GGATAA**GATGTGTTTCCAGATTGAC**TTATCCT**CACTAAA**GGATAAA**AC

<center>•   •   •   •   •   •
-100   -80   -60   -40   -20</center>

<center>HipB ⟶   *Bgl* II</center>

TTATAA**TATCCCCTTAAGCGGATA**AACTTGCTGTGGACGTATGACATGATGAGCTTTCAGAAGATCT

<center>•   •   •   •
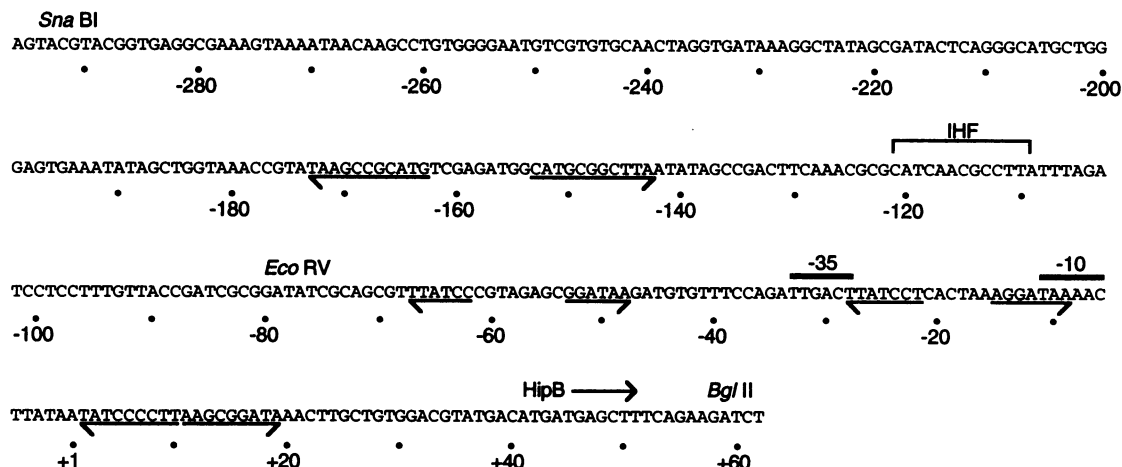+1   +20   +40   +60</center>

FIG. 1. Nucleotide sequence of the nontranscribed strand of the *hip* regulatory region (4). The sequence is numbered with respect to the *hip* transcriptional start site at +1. The −10 and −35 regions of the promoter are overlined. Regions of dyad symmetry are indicated with pairs of opposing arrows. The site that is similar to the IHF consensus binding site (11) is marked. Relevant restriction sites are noted.

hipA (4). A *polA* strain, NO3434, was transformed with pNO3075H, and Amp^s Cm^r colonies were obtained as described above. *hipA::cat* was transferred from HM2010 to the desired backgrounds, and the insertions were confirmed by Southern analysis with the *Hpa*I-*Cla*I fragment of *hip* as the probe. This probe detects the 1,345-bp *Bgl*II-*Cla*I fragment of the intact gene or the 2,297-bp fragment of the gene interrupted by *cat*.

hipB::cat. Attempts to interrupt *hipB* were unsuccessful. NO3434 was transformed with pNO3075A or pNO3075AR. These plasmids contain *cat* inserted in *hipB* at *Asu*II. Plasmid integrates, Amp^r Cm^r, were obtained, but these integrates could not be resolved, since Amp^s Cm^r strains were not recovered. This result, alone, suggests that *hipB* is essential for cell viability; however, in view of the fact that both *hipB* and *hipA* can be deleted from the chromosome, an alternative explanation is that *hipA* may be toxic to cells in the absence of a functional *hipB* (see Results).

Media. The culture media were LB broth (24), M9ZB broth (35), and TYE agar (4). For growth of *dapE6* strains, 75 mg of diaminopimelic acid per liter was added to these media. The media were supplemented with 40 mg of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside per liter to visualize β-galactosidase activity resulting from *hip-lacZYA* promoter fusions in cells on agar plates.

Unless otherwise specified, antibacterial agents were used in the following amounts per liter: ampicillin, 100 mg in LB broth and TYE agar but 50 mg in M9ZB broth or for strains with single copies of *bla*; chloramphenicol, 25 mg; kanamycin sulfate, 75 mg but 35 mg for strains with single copies of the kanamycin resistance (Kan^r) determinant; nitrofurantoin, 2 mg; and tetracycline HCl, 20 mg but 4 mg for Δ*hipBA::tetA* strains.

Determination of frequency of persistence. The frequencies of persistence to inhibition of DNA or peptidoglycan synthesis were determined by previously described methods (4, 25, 26).

Recombinant DNA methods. Subcloning procedures, including restriction digestion, gel electrophoresis, isolation and purification of DNA fragments, ligation, and transformation, were performed by the methods of Maniatis et al. (22). DNA fragments were 5' end labeled by using $[\gamma\text{-}^{32}P]ATP$ (5,000 Ci/mmol) and T4 polynucleotide kinase (22). Plasmid DNA was prepared by the method of Humphreys et al. (19). Southern blot analysis was done as previously described (1).

Construction of *hip-lac* operon fusions. HM352 containing the *hip-lac* operon fusion in single copy at the λ *att* site was constructed and verified by the methods of Simons et al. (32). P1 transduction was used to create Δ*hipBA::tetA* and *hipA::cat* derivatives (Table 1).

Measurement of β-galactosidase activity. Cultures grown overnight to saturation were diluted 1:100 into 5 ml of LB broth. Ampicillin was added to the medium as appropriate. The cultures were grown to an optical density at 550 nm of 0.30 to 0.40 and placed in an ice-and-water bath to stop growth. The cells were permeabilized with chloroform and sodium dodecyl sulfate (SDS) by vortexing for 2 s, and β-galactosidase activity was measured as described by Miller (24).

Protein analyses. SDS-polyacrylamide gel electrophoresis (PAGE) was performed as previously described (21). Gels were stained with Coomassie brilliant blue R-250 or with silver by using the Bio-Rad silver staining kit. Protein concentrations were measured by the method of Bradford (5) by using the Bio-Rad protein assay kit and bovine immunoglobulin G as the standard. The first six amino acid residues of HipB were determined by the Biotechnology Instrumentation Facility (University of California, Riverside) with a pulsed-liquid protein sequencer (Applied Biosystems model 475-A) equipped on-line with a microbore phenylthiohydantoin analyzer (model 120-A; Applied Biosystems) and data system (model 900-A). The native molecular weight of HipB was estimated by size exclusion chromatography with a calibrated column (1 by 120 cm) of Bio-Gel P-60 (Bio-Rad) equilibrated with MS+ buffer (50 mM Tris-HCl [pH 7.6], 100 mM NaCl, 1 mM EDTA, 5% [vol/vol] glycerol, 1 mM phenylmethanesulfonyl fluoride [PMSF], 5 mM β-mercaptoethanol) at 4°C.

Preparation of HipB and HipA. (i) HipB. Overexpression was achieved by the methods of Studier et al. (35). BL21(DE3) containing pET3RH was grown in 500 ml of M9ZB broth supplemented with ampicillin (50 μg/ml). When the cells reached an optical density at 600 nm of approximately 0.7, expression of *hipB* was induced with 0.4 mM isopropyl-β-D-thiogalactopyranoside (IPTG), and the cultures were grown for an additional 3 h. The cultures were split into 250-ml portions, and the cells were collected by centrifugation at 4,420 × *g* for 10 min at 4°C. The pellets were washed with ice-cold MS+ buffer, frozen in a dry ice-ethanol bath, and stored at −70°C.

All steps in the purification procedure were performed on

TABLE 1. Bacteria, bacteriophage, and plasmids

Bacterial strain, bacteriophage, or plasmid	Relevant characteristic(s)[a]	Reference, construction, or source
E. coli strains[b]		
BL21(DE3)	F⁻ *ompT* r$_B$⁻ m$_B$⁻ λ(*lacUV5*-T7 gene *1*)	35
HM22	*hipA7 zde-264*::Tn*10 dapE6*	25
HM352	Δ(*lac-pro*) λ352 lysogen (*hip-lacZYA*)Kan^r	λ352 × P90C
HM2004	*zdd-262*::IS*10* Cm^r IS*10 hipA7 zdd-263*::Tn*5 dapE6*	P1 · KF1270 × HM22
HM2005	*zdd-262*::IS*10* Cm^r IS*10 hipA7 polA1*	P1 · HM2004 × NO3434
HM2006	*zdd-262*::IS*10* Cm^r IS*10* Δ*hipBA*::*tetA polA1*	Materials and Methods
HM2010	*hipA*::*cat polA1*	Materials and Methods
HM5003	Δ(*lac-pro*) λ352 lysogen (*hip-lacZYA*)Kan^r *zdd-262*::IS*10* Cm^r IS*10* Δ*hipBA*::*tetA*	P1 · HM2006 × HM352
HM5004	Same as HM5003 but *hip*⁺	P1 · HM2006 × HM352
HM5010	Δ(*lac-pro*) λ352 lysogen (*hip-lacZYA*)Kan^r *hipA*::*cat*	P1 · HM2010 × HM352
HM5013	Δ(*lac-pro*) λ352 lysogen (*hip-lacZYA*)Kan^r *zdd-262*::IS*10* Cm^r IS*10* Δ*hipBA*::*tetA recA56 srlC300*::Tn*10*	P1 · JC10240 × HM5003[c]
HM5014	Same as HM5013 but *hip*⁺	P1 · JC10240 × HM5004[c]
JC10240	*recA56 srlC300*::Tn*10*	4
KF1270	*zdd-262*::IS*10* Cm^r IS*10 zdd-263*::Tn*5 zde-264*::Tn*10*	25
NO3434	*polA1* (spontaneous Str^r mutant of NO2383)	8
P90C	Δ(*lac-pro*)	G. W. Hatfield
Bacteriophages		
λRS88	Specialized phage for recombination with pRS vectors	32
λ352	λRS88 recombinant carrying *hip-lacZYA* operon fusion of pGSSB	This study
Plasmids		
pDB430	pNO1575 derivative with *Eco*RV-*Hpa*I fragment (−77 to +353) carrying *hip* promoter and *hipB* inserted downstream of *lac* promoter	4
pDB430a	pDB430 with a 2-bp insertion in *hipB* at *Asu*II[d]	4
pDB1661	Blunted *Afl*II-*Bsp*HI fragment (+9 to +1670) cloned into *Sma*I site of pNO1575, placing *hipB* and *hipA* downstream of *lac* promoter	This work
pDB1760	*Asu*II-*Bam*HI fragment (+175 to +1938) ligated to the *Acc*I- and *Bam*HI-digested pNO1575, placing *hipA* downstream of *lac* promoter	4
pET-3	T7 transcription vector; Amp^r	35
pET3AA	End-filled *Afl*II fragment (+9 to +1797) inserted into the unique *Bam*HI site (blunted) of pET-3 such that *hipB* and *hipA* are transcribed from the T7 promoter	This work
pET3RH	*Eco*RV-*Hpa*I (−77 to +353) fragment subcloned into *Bam*HI site of pET-3 such that *hipB* is transcribed from the T7 promoter	This work
pGK430	pKO11 derivative with *Eco*RV-*Hpa*I (−77 to +353) fragment carrying *hip* promoter and *hipB*	4
pGSSB	352-bp *Sna*BI-*Bgl*II fragment (−291 to +61), subcloned from pSP72SB as a 359-bp *Bam*HI-*Bgl*II fragment, inserted into *Bam*HI-digested pRSGN551. Orientation is such that a *hip-lac* transcriptional fusion is created; Amp^r Kan^r	This work
pHM418	Source of *hip* fragments for plasmid constructions	26
pKO11	Promoterless vector; Amp^r	4
pNO1575	pBR322 derivative containing the *lac* promoter and polylinker region of pUC9; Amp^r	4
pNO2295tet	pNO3075 with 788-bp *Eco*RV fragment of *hip* (−77 to +711) removed and replaced with 1,427-bp *Eco*RI-*Ava*I Tet^r fragment from pBR322; Amp^r Tet^r	This work
pNO3075	pNO1575 derivative with 3,095-bp *Bam*HI fragment from pHM418 containing *hip* operon; Amp^r	This work
pNO3075A	Insertion in *hipB* made by cleaving pNO3075 with *Asu*II and ligating to 952-bp *Asu*II fragment of pBR325, which contains *cat* (Cm^r). *hip* and *cat* in same orientation; Amp^r Cm^r	This work
pNO3075AR	pNO3075A with orientation of *cat* reversed	This work
pNO3075H	Insertion in *hipA* made by cleaving pNO3075 with *Hpa*I and ligating to 952-bp *Asu*II *cat* (Cm^r) fragment of pBR325. *hip* and *cat* in same orientation; Amp^r Cm^r	This work
pRS551	*lacZYA* transcriptional fusion vector; Amp^r Kan^r	32
pRSGN551	pRS551 derivative containing multiple cloning region of pUC18 upstream of *lacZ*. Constructed by inserting the *Eco*RI-*Hind*III (*Hind*III end-filled) fragment of pUC18 into the *Eco*RI and *Bam*HI (end-filled) sites of pRSGN551.	G. W. Hatfield
pSP72	Cloning vector; Amp^r	Promega
pSP72SB	*Sna*BI-*Bgl*II fragment (−291 to +61) ligated into the *Sma*I- and *Bgl*II-digested pSP72	This work
pSP72TA	*Taq*I-*Asu*II fragment (−161 to +176) inserted into *Acc*I-digested pSP72 such that the *Acc*I-*Taq*I junction is immediately downstream of the *Hind*III site in pSP72 polylinker	This work

[a] *hip* fragments of plasmids are numbered with respect to the *hip* transcriptional start site at +1 (Fig. 1) (4).

[b] Strains are all *E. coli* K-12 except for BL21(DE3), which is an *E. coli* B strain.

[c] Strains containing Δ*hipBA*::*tetA* are resistant to low levels of tetracycline (4 μg/ml) but sensitive to high levels, making it possible to select for Tn*10* recipients with medium containing 40 μg of tetracycline per ml. To confirm the presence of *recA*, the transductants were scored for sensitivity to nitrofurantoin (2 μg/ml).

[d] The 2-bp insertion at *Asu*II creates a frameshift in *hipB* and results in a stop codon 68 bp downstream. A truncated protein can be visualized in vitro (4).

ice or at 4°C. Frozen cell pellets (approximately 4 g [wet weight]) were thawed on ice and resuspended in 11 ml of ice-cold MS+ buffer. The resuspended cells were ruptured in an Aminco French pressure cell at a pressure of 1,000 lb/in². The lysate was clarified by centrifugation at 12,000 × g for 30 min at 4°C, and the supernatant was then centrifuged at 105,000 × g for 90 min at 4°C. HipB remained soluble and was found in the supernatant of this crude extract (fraction I, typically 9 ml).

Fraction I was applied to a DEAE-5PW fast protein liquid chromatographic (FPLC) column (8.0 mm by 7.5 cm; Millipore) equilibrated with buffer A (20 mM Tris-HCl [pH 7.6]). After the column was washed with 10 ml of the buffer, bound proteins were eluted at 1 ml/min with an 80-ml linear gradient from 0 to 0.5 M NaCl in buffer A. SDS-PAGE analysis of the fractions revealed that HipB eluted from the column between 0.19 and 0.26 M NaCl. Fractions containing HipB were pooled, and the pH was adjusted to pH 6.5 with HCl (fraction II, typically 12 ml).

Fraction II was applied to an SP-5PW FPLC column (8.0 mm by 7.5 cm; Millipore) equilibrated with buffer B (20 mM potassium phosphate [pH 6.5]). The column was washed with 10 ml of this buffer, and bound proteins were eluted at 1 ml/min with a 80-ml linear gradient from 0 to 1 M NaCl in buffer B. HipB eluted as a symmetrical peak at a NaCl concentration of 0.40 M. Fractions containing HipB were pooled and concentrated by ultrafiltration with an Amicon Centriprep-3 concentrator (fraction III, typically 0.5 ml).

Fraction III was applied to a column (1 by 50 cm) of Sephacryl S-300 (Sigma) equilibrated with MS+ buffer. The sample was eluted with the same buffer at a flow rate of 8 ml/h. Fractions of approximately 1 ml were collected and analyzed by SDS-PAGE. HipB was typically found in fractions 23 to 28. These fractions were pooled and concentrated approximately 10-fold with an Amicon Centriprep-3 concentrator (fraction IV, typically 0.6 ml). After this last step of the purification procedure, HipB was greater than 95% pure on the basis of densitometric scanning of Coomassie blue-stained gels. Typical yields were 2 to 3 mg of pure protein per 250 ml of culture. HipB was stored in small aliquots at −70°C with no appreciable loss of DNA-binding activity for at least 6 months.

(ii) HipA. Overexpression and extraction of HipA were done by the procedures described above for HipB, except that BL21(DE3) contained pET3AA, and the pellets were washed with S+ buffer (50 mM Tris-HCl [pH 7.6], 50 mM NaCl, 1 mM EDTA, 5% [vol/vol] glycerol, 1 mM PMSF, 5 mM β-mercaptoethanol). HipA, as well as HipB, was found in the supernatant of the crude extract (fraction I, typically 10 ml). This crude extract contained approximately 7% HipA and 7% HipB.

Fraction I was applied to an SP-5PW FPLC column (8.0 mm by 7.5 cm; Millipore) equilibrated with buffer B. The column was washed with 10 ml of the buffer, and bound proteins were eluted at 1 ml/min with an 80-ml linear gradient from 0 to 1 M NaCl in buffer B. Two major protein peaks were detected by monitoring at 214 nm. SDS-PAGE analysis of the fractions indicated that HipA eluted from the column between 0.48 to 0.55 M NaCl. All of the HipA fractions contained some HipB, although the majority of HipB eluted earlier between 0.35 to 0.45 M NaCl. Fractions containing HipA were pooled and concentrated by ultrafiltration using an Amicon Centriprep-3 concentrator (fraction II, HipA-HipB complex, typically 0.5 ml). On the basis of densitometric scanning of Coomassie blue-stained gels, this preparation contained approximately 65% HipA and 12% HipB. Since HipA is about five times the size of HipB, the HipA-HipB complex contains equimolar amounts of each protein.

Fraction II was denatured by adding urea to a final concentration of 6 M. This preparation was applied to a column (1 by 50 cm) of Sephacryl S-300 equilibrated with S+ buffer containing 6 M urea. The sample was eluted with the same buffer at a flow rate of 6 ml/h. Fractions of approximately 1 ml were collected and analyzed by SDS-PAGE. HipA was typically found in fractions 14 to 18. These fractions were pooled and dialyzed against S+ buffer overnight with two 500-ml changes, using SPECTRA/POR membrane with a molecular weight cutoff of 12,000 to 14,000 (Spectrum Medical Industries). A copious precipitate formed and was collected by centrifugation at 17,400 × g for 30 min at 4°C. The pellet, which contained the majority of HipA, was resuspended in 1 ml of S+ buffer containing 0.25% sodium lauroyl sarcosinate (Sarkosyl) (CIBA-GEIGY). This suspension was centrifuged as described above, and analysis by SDS-PAGE indicated that the vast majority of HipA remained in the supernatant (fraction III, HipA).

Gel retardation assays. Binding of proteins to the *hip* regulatory region was measured by the polyacrylamide gel retardation assay (12, 14). DNA fragments were 5′ end labeled and added to binding buffer D (10 mM Tris-HCl [pH 7.6], 50 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 0.24 mg of BSA per ml, 4% [vol/vol] glycerol, 2.4 μg of sonicated salmon sperm per ml) at a concentration of less than 10^{-9} M. Salmon sperm DNA was not included in experiments estimating the K apparent (K_{app}) of HipB, since extremely small amounts of probe (1 pM) were used in these studies, and HipB binding was specific in the protein range tested (see Results). Protein preparations were freshly diluted in binding buffer D and added to the mixture last. The final volume of the binding reaction mixture was 15 μl. Following a 15-min incubation at room temperature (22 to 25°C), 1.5 μl of 10× loading buffer (50% glycerol, 0.4 M Tris-HCl [pH 7.6], 0.125% bromophenol blue, 0.125% xylene cyanol) was added to the tube. Samples were loaded onto a native polyacrylamide gel and electrophoresed in low-ionic-strength running buffer (6.7 mM Tris-HCl [pH 7.6], 1 mM EDTA, 3 mM sodium acetate). The running buffer was recirculated between compartments. After electrophoresis, the gels were dried under vacuum, and the DNA fragments were visualized by autoradiography. Binding was quantified by using an LKB Ultroscan II laser densitometer.

The binding activity of purified preparations of HipB was determined as described by Riggs et al. (29). On the basis of the experimental data (see Results) that four HipB dimers bind one DNA molecule, HipB preparations were 80 to 96% active. In this work HipB concentrations are corrected for activity and are based on a monomeric molecular weight of 10,000.

Fragments used in the DNase I and methylation protection experiments. For analysis of the transcribed DNA strand of the *hip* regulatory region, the 389-bp *Hin*dIII-*Bgl*II restriction fragment from pSP72SB was 5′ end labeled and then digested with *Bam*HI to generate the 359-bp *Bam*HI-*Bgl*II fragment uniquely labeled at the *Bgl*II site (bp +61). This fragment was purified by PAGE and quantified by ethidium bromide fluorescence (22).

For analysis of the nontranscribed DNA strand of the *hip* promoter region, the 395-bp *Hin*dIII-*Cla*I fragment from pSP72TA was 5′ end labeled and cleaved with *Bam*HI to yield a 369-bp *Hin*dIII-*Bam*HI fragment uniquely labeled at the *Hin*dIII site from the pSP72 polylinker cloning region.

DNase I protection. DNase I protection studies employed the methods of Galas and Schmitz (13) and Johnson et al. (20). A uniquely 5′-end-labeled restriction fragment (3.2 × 10^{-11} final concentration) containing the *hip* regulatory region was incubated with an increasing amount of protein in a 50-μl

reaction mixture of footprinting buffer (10 mM Tris-HCl [pH 7.6], 50 mM NaCl, 0.1 mM EDTA, 1 mM dithiothreitol, 100 μg of BSA per ml, 2.5 μg of sonicated salmon sperm per ml, 4% [vol/vol] glycerol, 2.5 mM MgCl$_2$, 1 mM CaCl$_2$). After 15 min at 25°C, 1.4 μl (0.005 U) of diluted DNase I was added to the mixtures and incubation was continued for another 5 min. The reactions were then quenched by the addition of 184 μl of an ice-cold solution of 92% ethanol, 0.54 M ammonium acetate, and 71 μg of tRNA per ml. After briefly vortexing at high speed, the samples were immediately placed in a dry ice-ethanol bath to precipitate the DNA. The DNA was pelleted in a microcentrifuge for 15 min at 4°C, washed twice with 1 ml of ice-cold 70% ethanol, and briefly dried under vacuum. The pellets were resuspended in 5 μl of a formamide dye solution (80% [vol/vol] formamide, 10 mM sodium hydroxide, 1 mM EDTA, 0.1% xylene cyanol, 0.1% bromophenol blue), heated for 3 min at 90°C, and quickly chilled on ice. Samples were loaded onto a 6% polyacrylamide (19:1 acrylamide to bis ratio), 7 M urea sequencing gel in TBE buffer (90 mM Tris, 90 mM boric acid, 2.5 mM EDTA). After electrophoresis, the gels were covered with plastic wrap and exposed to X-ray film. In order to determine the nucleotide sequences protected from DNase I digestion, the samples were electrophoresed next to a sequencing ladder produced by G>A chemical cleavage (23) of the corresponding labeled DNA fragment.

Dimethyl sulfate protection. Dimethyl sulfate protection experiments were performed essentially as described by Siebenlist and Gilbert (31). The appropriate DNA fragment, uniquely 5' end labeled, was added to 100-μl reaction mixtures at a final concentration of approximately 0.15 nM. The reaction mixtures contained footprinting buffer plus 50 mM sodium cacodylate. A saturating amount of purified HipB (26 nM final concentration) was added, and binding proceeded for 15 min at 25°C. The mixture was cooled to 0°C, and 1 μl of dimethyl sulfate (Aldrich) was added. After 10 min at 0°C, the reaction was quenched by the addition of 25 μl of a solution of 1.5 M sodium acetate (pH 7.0), 1.0 M β-mercaptoethanol, and 500 μg of tRNA per ml. The DNA was precipitated with ethanol and treated according to the G>A chemical cleavage reaction methods of Maxam and Gilbert (23). A portion of the sample (approximately 20,000 cpm) was analyzed by electrophoresis as described for the DNase I protection studies. As controls, DNA in the absence of protein was treated in an identical manner, and all reactions were performed at least twice.

DNA sequence analysis. A FASTA search (28) of GenBank, version 66, was conducted to search for DNA and protein sequences similar to *hip*. Russell Doolittle (University of California, San Diego) also inspected the *hip* sequence using his repertoire of computer programs.

RESULTS

Disruptions in the *hip* locus. Deletions and insertions were created in *hip* (Materials and Methods) as part of a strategy to examine the regulation of the *hip* promoter. Strains in which *hipA* or both *hipA* and *hipB* had been inactivated were readily recovered. Unlike the earlier *hip* mutants (4, 25), these null mutants have normal growth at 25 and 37°C and parental frequencies of persistence (10^{-6} to 10^{-5}). Strains in which *hipB* but not *hipA* had been disrupted could not be recovered. The latter observation is probably related to the observation that strains defective in or lacking *hipB* cannot be transformed by *hipA*-containing plasmids (4). Thus, HipA appears to be toxic in the absence of HipB, and an interaction between these gene products might be anticipated. Indeed, biochemical evidence for such an interaction will be presented below.

TABLE 2. Effects of *hip* plasmids on expression from the *hip-lac* fusion in HM5013 and HM5014

Strain	Plasmid	Description[a]	β-Galactosidase activity[b]
HM5013 (Δ*hipBA*)	pNO1575	Vector	466.2 ± 13.1
	pKO11	Vector	429.0 ± 2.0
	pGK430	P$_{hip}$ *hipB*	4.5 ± 0.6
	pDB430	P$_{lac}$ P$_{hip}$ *hipB*	<1.0
	pDB430a	2-bp insertion	469.5 ± 15.3
	pDB1760	P$_{lac}$ *hipA*	NA[c]
	pDB1661	P$_{lac}$ *hipBA*	<1.0
HM5014 (*hip*[+])	pNO1575	Vector	28.3 ± 0.3
	pDB430	P$_{lac}$ P$_{hip}$ *hipB*	<1.0
	pDB1760	P$_{lac}$ *hipA*	22.5 ± 1.3
	pDB1661	P$_{lac}$ *hipBA*	<1.0

[a] pNO1575 is the vector for the pDB plasmid series, and pKO11 is the vector for pGK430. See Table 1 for descriptions of plasmids.
[b] β-Galactosidase activity was determined as described in Materials and Methods and is expressed in Miller units (24). Values represent the averages ± standard deviations of three separate experiments, each performed in duplicate.
[c] NA, not applicable.

Activity of *hip* promoter-*lacZ* fusions. The regulation of the *hip* promoter was first examined by measuring the effect of a deletion in the *hip* locus on the β-galactosidase activities of strains containing a transcriptional fusion of the *hip* regulatory region (bp −291 to +61) and *lacZ* inserted in single copy at the λ *att* site. The β-galactosidase activity, and by inference *hip* promoter activity, was 16-fold higher in HM5003, the strain containing the *hip* deletion, than in HM5004, the corresponding *hip*[+] strain. Since both *hipA* and *hipB* had been inactivated by the deletion, this result suggested that the *hip* promoter is regulated by one or both *hip* gene products.

The roles of *hipB* and *hipA* in the regulation of the *hip* promoter were analyzed in *recA* derivatives of the fusion-containing strains (Table 2). Promoter activity in the Δ*hipBA* strain was reduced approximately 95-fold by a plasmid expressing *hipB* under the control of the *hip* promoter, pGK430. When *hipB* was placed under the control of the *lac* promoter, pDB430, *hip* promoter activity was reduced to below the limits of accurate measurement. A drastic reduction was also observed in the corresponding *hip*[+] strain. Repression was eliminated by a 2-bp insertion in *hipB*, pDB430a.

The effect of *hipA* on the regulation of the *hip* promoter is less marked. pDB1760, which expresses *hipA* from the *lac* promoter, reduced *hip* promoter activity of the *hip*[+] strain slightly (Table 2). Unfortunately, the effect of *hipA* in *trans* on *hip* promoter activity cannot be assessed in the Δ*hipBA* strain, since such strains cannot be transformed with plasmids expressing *hipA*, even when expression is minimized by inserting the *hipA* open reading frame at vector sites far from recognized promoters (4). Expression of both *hipB* and *hipA* from the *lac* promoter, pDB1661, reduced the activity of the *hip* promoter to the same extent as did a plasmid expressing only *hipB* from the *lac* promoter, pDB430 (Table 2).

Insertional inactivation of *hipA* also suggested that the *hipA* gene product has a slight repressive effect on the *hip* promoter. Promoter activity in HM5010, *hipA::cat*, was increased 1.7-fold over that of the corresponding *hip*[+] strain, HM352. This increase in promoter activity caused by inactivation of *hipA* was eliminated by supplying *hipA* in *trans*.

It has not been possible to use similar in vivo analysis to determine whether *hipA* has a role independent of *hipB* in the regulation of the *hip* promoter because, as noted above, strains

in which *hipB* has been interrupted and *hipA* is intact cannot be recovered.

Properties of HipB. HipB was overexpressed and purified as described in Materials and Methods. The first six amino acid residues, MMSFQK, were identical to those predicted from the DNA sequence (4).

The subunit molecular weight of HipB, as determined by SDS-PAGE, is approximately 10,000, which is in close agreement to the value inferred from the nucleotide sequence of *hipB* (4). A native molecular weight of approximately 26,000 was obtained by gel filtration chromatography with Bio-Gel P-60. Cross-linking studies were performed by the method of Staros (34) using 3,3'-dithiobis(sulfosuccinimidyl propionate) and HipB, which had been purified omitting β-mercaptoethanol from the last step. These studies showed that HipB forms dimers in solution and that the dimeric state is not caused by disulfide bonds between the cysteine residues of the monomers.

Although the predicted amino acid sequence of *hipB* does not bear extensive resemblance to any other protein (4), a portion of the sequence is highly similar to the helix-turn-helix DNA-binding motif found in many prokaryotic regulatory proteins. Statistical analysis using the methods of Brennan and Matthews (6) and Dodd and Egan (9), as well as computer analysis (10), support the view that HipB contains a helix-turn-helix motif near the N terminus and is a Cro-like, DNA-binding protein.

Gel retardation of DNA fragments of the *hip* regulatory region by HipB. Analysis of the interaction of HipB with the *hip* regulatory region was conducted with the following ^{32}P-labeled DNA fragments: the *Bam*HI-*Bgl*II fragment of pSP72SB, which contains bases −291 through +61 of *hip*; the *Eco*RV-*Bgl*II fragment of pSP72SB, which contains bases −77 through +61 of *hip*; and the *Bam*HI-*Eco*RV fragment of pSP72SB, which contains bases −291 through −78 of *hip* (Fig. 1).

The electrophoretic mobility of the *Bam*HI-*Bgl*II fragment, which contains the regulatory region of *hip*, was retarded by purified HipB (Fig. 2A). Only a single, retarded complex was observed. Additional experiments confirmed that this was the case even if the *Bam*HI-*Bgl*II fragment was titrated over a 100-fold range of protein concentrations and if the binding reaction mixtures were electrophoresed for longer times. Competition analyses using the unlabeled *Bam*HI-*Bgl*II fragment or salmon sperm DNA demonstrated that binding was specific (data not shown).

The amount of active protein required to obtain half-maximal binding of the DNA approximates the magnitude of K_{app}, provided the DNA concentration is negligible compared with the protein concentration at midpoint. A binding curve (Fig. 2B) was produced on the basis of densitometric scanning of the autoradiograph. Quantitation was based on reduction of the free DNA, rather than the appearance of the complex because of the apparent instability of the DNA-protein complex during electrophoresis. On the basis of the binding curve, 40-pM active HipB protein was required for half-maximal binding. This estimate of K_{app} is an approximation that assumes a binding stoichiometry of four HipB dimers per DNA molecule. The basis for this assumption will be presented in subsequent sections.

To further define the region of binding, the *Bam*HI-*Bgl*II fragment was cleaved at the *Eco*RV site of the *hip* regulatory region. This cleavage separates the upstream inverted repeat sequence from the three similar inverted repeat sequences (Fig. 1). HipB retarded the *Eco*RV-*Bgl*II fragment but failed to retard the *Bam*HI-*Eco*RV fragment even at a 100-fold-greater
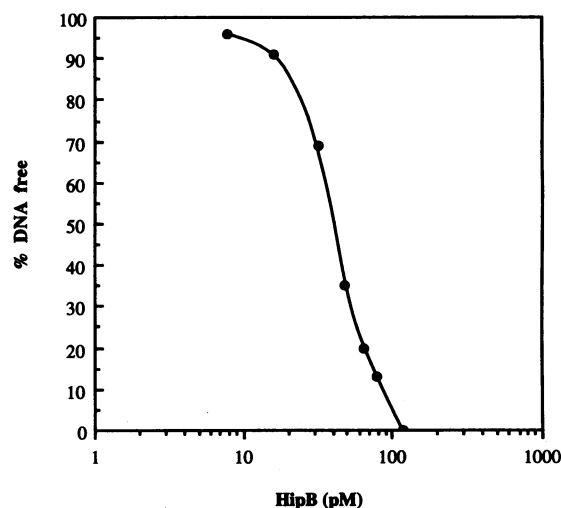
A
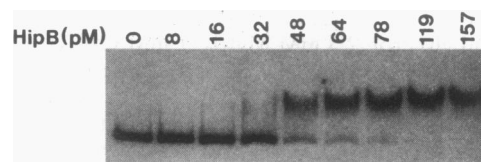
B

FIG. 2. Binding of HipB to the *hip* regulatory region. (A) Approximately 1×10^{-12} M of the 5'-end-labeled *Bam*HI-*Bgl*II restriction fragment from pSP72SB was incubated with increasing amounts of purified HipB in binding buffer (without salmon sperm DNA). Complexed DNA was separated from free DNA by electrophoresis in a 6.5% polyacrylamide gel. Reaction mixtures depicted in the lanes contained the indicated concentrations of active HipB. (B) Quantitation of gel retardation assay. The peak area values of the free DNA in the autoradiograph displayed above were determined from a linear range of multiple film exposure times, and the percentage of free DNA was plotted.

concentration. Although the *Bam*HI-*Eco*RV fragment is itself unable to bind HipB, removal of this region from the *Bam*HI-*Bgl*II fragment reduces HipB binding. HipB binds to the *Eco*RV-*Bgl*II fragment with a K_{app} of 0.4 nM, 10 times the amount required to half-bind the *Bam*HI-*Bgl*II fragment under identical reaction conditions (data not shown).

Effect of HipB on DNase I digestion and methylation of the *hip* regulatory region. HipB protected two regions on the transcribed strand from DNase I digestion (Fig. 3). Each region was approximately 50 residues long. One region extended from +21 to −34, and the other extended from −48 to −98. A similar protection pattern was observed on the nontranscribed strand (data not shown); one region of protection extended from +25 to −28 and another extended from −42 to −96. The two large regions of protection on the transcribed and nontranscribed strands were separated by 13 consecutive residues which remained susceptible to DNase I digestion. Protection by HipB was not uniform within the protected regions, since some phosphodiester bonds remained accessible to DNase I cleavage. There were no obvious sites of hypersensitive cleavage caused by the presence of HipB.

When the results of the DNase I protection studies are summarized (Fig. 4), it can be seen that the −10 and −35
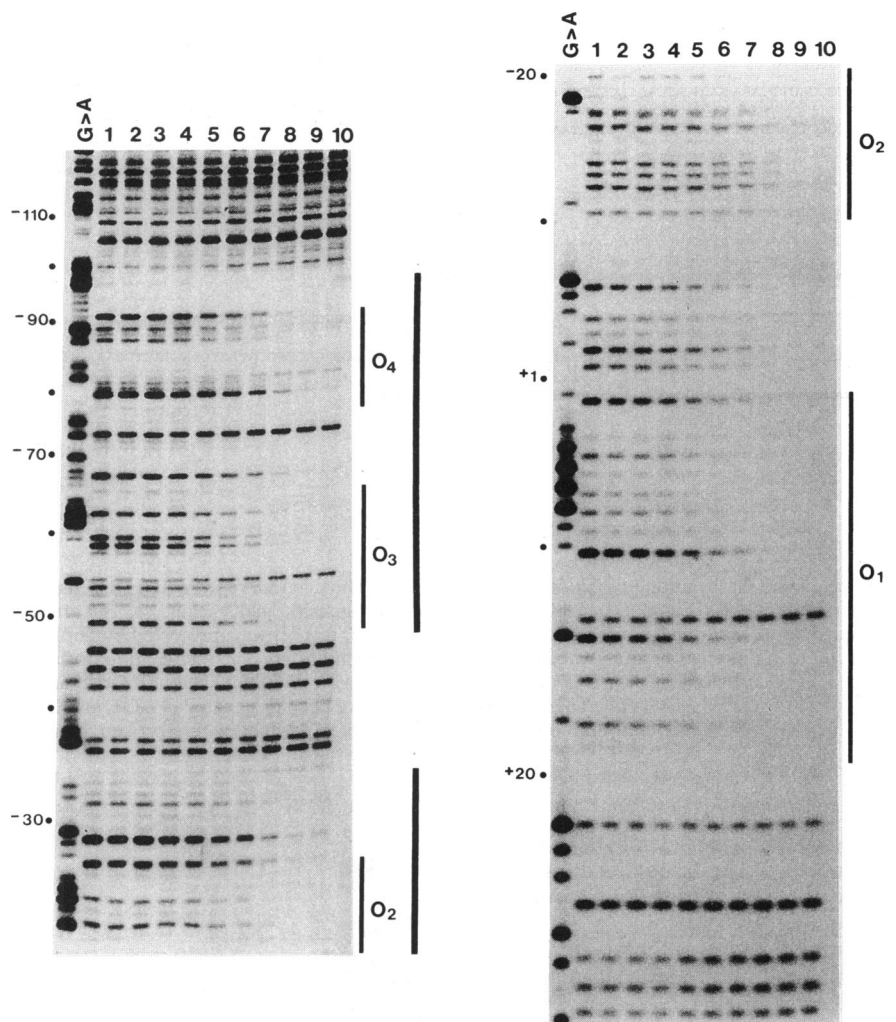
FIG. 3. DNase I protection of the transcribed strand of the *hip* regulatory region by HipB. The 359-bp *Bam*HI-*Bgl*II restriction fragment from pSP72SB (3.2×10^{-11} M) labeled at the *Bgl*II site was incubated with the following concentrations (nanomolar) of HipB: 0 (lanes 1), 0.1 (lanes 2), 0.2 (lanes 3), 0.4 (lanes 4), 0.8 (lanes 5), 1.5 (lanes 6), 3.1 (lanes 7), 6.2 (lanes 8), 12.4 (lanes 9), and 25.0 (lanes 10). The mixtures were subjected to partial DNase I digestion and then run adjacent to the Maxam and Gilbert G>A sequencing reaction (23) of the labeled probe. After electrophoresis, the gel was cut in half and exposed to film. The relevant part of the top half of the gel is shown on the left, and the bottom half of the gel is shown on the right. The sequence is numbered as shown in Fig. 1. The approximate extent of protection by HipB is indicated by the thick vertical lines. The thin vertical lines mark the locations of the O1, O2, O3, and O4 inverted repeats (see text).

regions of the *hip* promoter were protected from DNase I digestion by HipB. In addition, the areas protected by HipB encompass the three inverted repeats with the conserved motif, TATCCN$_8$GGATA. These sequences have been designated O1, O2, and O3. Protection extended 2 or 8 bp downstream of O1 but 30 or 32 bp upstream of O3, depending upon which DNA strand was examined. Closer analysis of the protected region upstream of O3 revealed a degenerate version of the inverted repeat sequence, designated O4. It should be noted that O4 is upstream of the *Eco*RV restriction site.

All four operators were half-maximally protected from DNase I digestion by similar concentrations of HipB, although the affinity of HipB for O2 may be slightly lower than those for the other three sites (Fig. 3, lanes 4 and 5).

To determine whether HipB interacted with the inverted repeat sequences, methylation protection studies were performed (Fig. 5A and B), and the results are summarized in Fig. 4. The transcribed strand contains 12 guanine residues which

were protected by HipB from methylation. In addition, HipB enhanced the methylation of 2 guanine residues and 5 adenine residues. All of the 19 affected residues were part of the inverted repeat sequences designated O1, O2, O3, and O4. A similar pattern of methylation protection and enhancement was observed with the nontranscribed strand. It was unclear whether HipB affected the methylation of the adenine at position −79, because this band could not be resolved from the adjacent guanine bands at positions −80 and −81.

Interaction of HipA with the *hip* regulatory region. Overproduction of HipA independently of HipB could not be achieved by using the pET-3 transcription vector. However, substantial amounts of HipA were recovered with a pET-3 derivative containing the coding regions of both *hipB* and *hipA*. HipA was found exclusively in a HipA-HipB complex composed of equimolar amounts of the two proteins. The complex could not be resolved by nondenaturing methods. Nearly complete resolution was obtained by gel filtration in 6 M urea

EcoRV

5'-GCGGCTTAATATAGCCGACTTCAAACGCGCATCAACGCCTTATTTAGATCCTCCTTTGTTACCGATCGCGGATATCGCAGCGTTTATCCCGTAGAGCGGATAAGATGTG

O4 →   O3 →

3'-CGCCGAATTATATCGGCTGAAGTTTGCGCGTAGTTGCGGAATAAATCTAGGAGGAAACAATGGCTAGCGCCTATAGCGTCGCAAATAGGGCATCTCGCCTATTCTACAC
   -140   -120   -100   -80

-35 ____   -10 ____
TTTCCAGATTGACTTATCCTCACTAAAGGATAAAACTTATAATATCCCCTTAAGCGGATAAACTTGCTGTGGACGTATGACATGATGAGCTTTCAGAAGATCT-'3

O2 →   O1 →

AAAGGTCTAACTGAATAGGAGTGATTTCCTATTTTGAATATTATAGGGGAATTCGCCTATTTGAACGACACCTGCATACTGTACTACTCGAAAGTCTTCTAGA-'5
-40   -20   +1   +20   +40   +60

Conserved   5'- TATCCNNNNNNNNNGGATA -3'
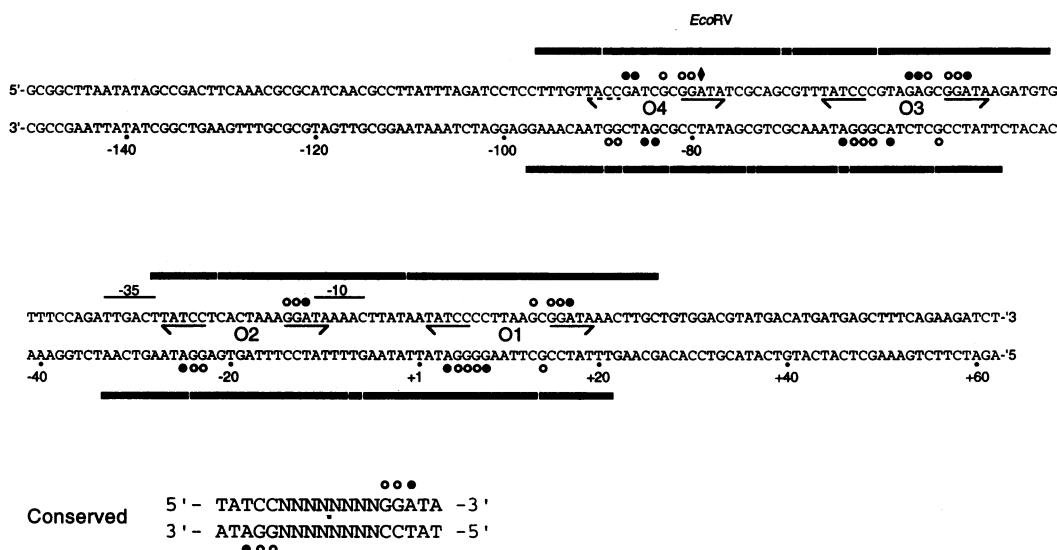   3'- ATAGGNNNNNNNNNCCTAT -5'

FIG. 4. Summary of the DNase I and methylation protection studies with HipB. The diagram represents a consensus protection pattern derived from a number of experiments. The sequence is numbered with respect to the *hip* transcriptional start at +1. The areas overlined and underlined with a heavy bar represent sequences of the nontranscribed and transcribed strands, respectively, protected by HipB against DNase I digestion. Bases protected (●) or enhanced (•) in dimethyl sulfate protection experiments are indicated. The methylation profile of the adenine at position −79 was unclear (◆). The −10 and −35 regions of the *hip* promoter are overlined with thin lines. The horizontal arrows indicate the conserved bases of the inverted repeat sequence TATCCN₈GGATA. These inverted repeats have been designated O1, O2, and O3. The sequence designated O4 is a degenerate version of this inverted repeat, and the nonconserved half-site is underlined with a broken arrow. A conserved methylation protection pattern is presented.

(see Materials and Methods for further details). Unfortunately, even the best preparations of HipA contained traces of HipB on the basis of observation of overloaded, Coomassie blue-stained SDS-polyacrylamide gels.

Whereas only one retarded band was observed in gel retardation assays with HipB, multiple retarded bands were formed with the HipA-HipB complex (Fig. 6). On the basis of electrophoretic mobility, the faint CI band corresponds to the retarded band observed with HipB alone and might represent binding by HipB which has become dissociated from the HipA-HipB complex. The CII, CIII, and CIV bands are more highly retarded, suggesting that these bands result from the binding of HipA, directly or indirectly, via HipB.

In order to distinguish between these alternatives, a preparation of HipA free of HipB would be desirable. As noted above, such a preparation has not been obtained. Therefore, gel retardation assays were performed with a HipA preparation containing traces of HipB (Fig. 7A). Large amounts of the HipA preparation were required for band shifts. However, binding was specific, as judged by competition studies using excess molar amounts of unlabeled vector, pSP72, or vector containing the *hip* regulatory region, pSP72SB (data not shown). Because traces of HipB are present, it is possible that the active binding species is the HipA-HipB complex rather than HipA itself. Indeed, the binding of the HipA preparation was increased by even a small increment of HipB (Fig. 7B). With larger amounts of HipB, more highly retarded bands were observed. With still larger amounts of HipB, a more rapidly migrating band predominated, corresponding in mobility to that of the band formed with HipB alone.

The HipA-HipB complex binds to the *hip* regulatory region with an affinity similar to that of HipB alone. Approximately 0.07 μg of the HipA-HipB complex per ml was required to half-bind the *Bam*HI-*Bgl*II fragment (Fig. 6). This corresponds to 0.008 μg/ml of HipB, assuming that the HipA-HipB prep-
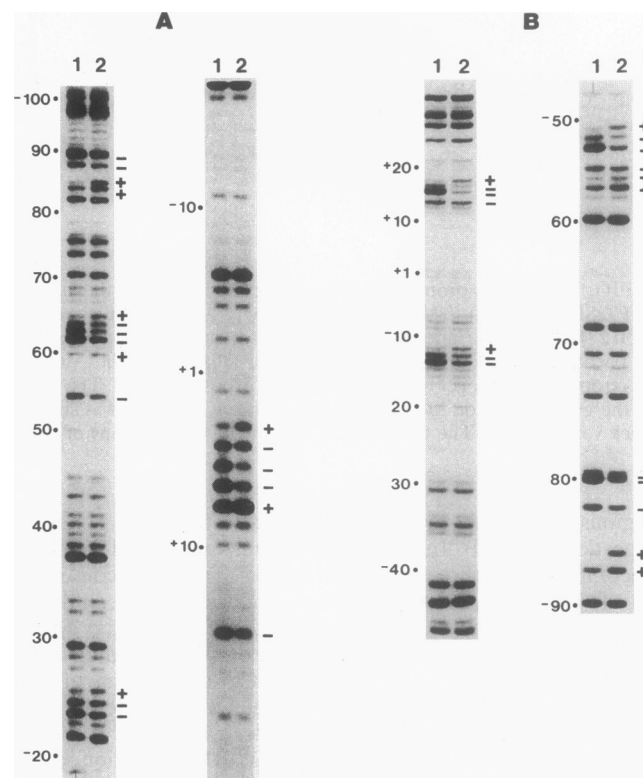

FIG. 5. HipB interaction with specific purine residues. Autoradiograms displaying the methylation protection patterns of the transcribed (A) and nontranscribed strand (B) of the *hip* regulatory region in the absence (lanes 1) and presence (lanes 2) of a saturating amount of HipB (see Materials and Methods). The purine residues that were protected from methylation (−) and the purine residues whose methylation was enhanced in the presence of HipB on the basis of densitometric scanning (+) are indicated to the right of the gels.
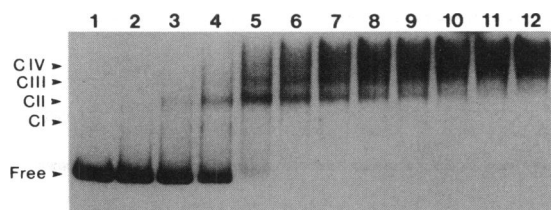
FIG. 6. Gel retardation assay with HipA-HipB complex. Increasing amounts of HipA-HipB were added to binding reaction mixtures containing the end-labeled *Bam*HI-*Bgl*II restriction fragment (3.2×10^{-11} M). Complexed DNA was separated from free DNA by electrophoresis in a 6.5% polyacrylamide gel. Lanes 1 to 12 contained 0, 0.02, 0.04, 0.07, 0.13, 0.20, 0.26, 0.34, 0.40, 0.53, 0.67, and 0.80 μg/ml, respectively, of HipA-HipB complex (fraction II, see Materials and Methods).

aration is fully active and that HipB represents 12% of this preparation (see Materials and Methods). A similar concentration of HipB (0.004 to 0.008 μg/ml) was needed to retard the same quantity of DNA under identical binding conditions (data not shown).

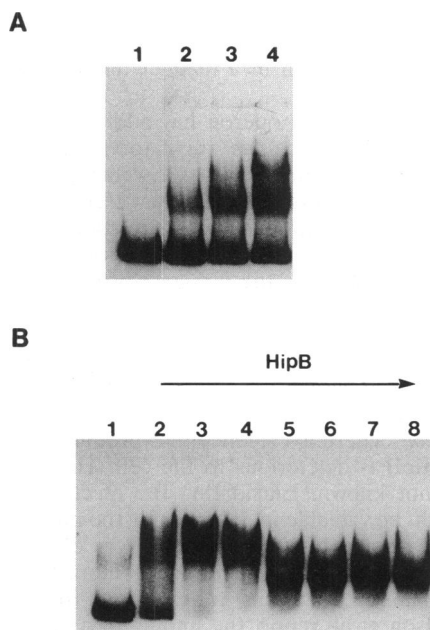**Effect of the HipA-HipB complex on DNase I digestion of the**

A

B

FIG. 7. (A) Binding activity of the HipA preparation. The end-labeled *Bam*HI-*Bgl*II restriction fragment (4×10^{-11} M) was incubated in binding buffer with the following final concentrations of HipA preparation (fraction III, Materials and Methods): 0 (lane 1), 3.5 (lane 2), 7 (lane 3), and 11 (lane 4) μg/ml. Samples were analyzed on a 5% polyacrylamide gel and visualized by autoradiography. (B) Effect of HipB on the binding activity of the HipA preparation. An increasing amount of purified HipB was added to a constant amount of HipA (fraction III). The protein mixtures were incubated on ice for 30 min and then added to tubes containing the labeled *Bam*HI-*Bgl*II fragment (5×10^{-11} M final concentration) in binding buffer. Samples were incubated for 15 min at room temperature and then electrophoresed in a 5% polyacrylamide gel. The final concentration of the HipA preparation in each reaction was 3.5 μg/ml. The final concentration of HipB in each reaction mixture was 0, 0.013, 0.026, 0.053, 0.13, 0.26, 0.53, and 1.3 μg/ml for lanes 1 to 8, respectively.

hip **regulatory region.** The interaction of the HipA-HipB complex with the *hip* regulatory region was examined by DNase I protection studies. The results are summarized and compared with the protection pattern obtained with purified HipB (Fig. 8). The HipA-HipB footprint is slightly larger, and eight sites were made hypersensitive to DNase I digestion by HipA-HipB but not by HipB. All four operators were half-maximally protected from DNase I digestion at similar concentrations of HipA-HipB (approximately 0.05 to 0.1 μg/ml). Thus, the appearance of multiple retarded bands in gel retardation assays may arise as a result of larger protein oligomers binding to the operators.

DISCUSSION

There are a number of indications that expression of *hip* is low and that the amount of HipA free in the cell is carefully regulated. First, the codon usage in *hip* exhibits a pattern often associated with weakly expressed operons (4). Second, the activity of the *hip* promoter in log-phase cells, as measured from single-copy *hip-lac* operon fusions inserted at the λ *att* site, is approximately 30 Miller units when the *hip* locus is intact. This value is similar to that obtained for the relatively weak IS*10*R-*p*IN promoter, which in single copy, is transcribed approximately 0.25 times per generation (32). HipB, a Cro-like protein on the basis of its inferred amino acid sequence, is a repressor of *hip* and chiefly responsible for the low level of *hip* promoter activity. Third, overexpression of both *hipB* and *hipA* by using the Studier T7 expression system (35) results in a protein extract in which HipA is found exclusively associated with HipB in an equimolar ratio. This association is tight, requiring denaturing conditions to separate the proteins. Furthermore, HipB is present in this extract at approximately five times the molar amount of HipA. Translational coupling of *hipB* and *hipA*, suggested by the sequence of the operon (4), may be responsible for setting this ratio. The multiple mechanisms for maintaining a low level of expression of the operon and little or no free HipA in the cell are probably related to the toxicity of HipA in the absence of HipB.

The identification of HipB as a repressor was suggested by the finding that *hipB* in *trans* can reduce the activity of the *hip* promoter to barely detectable levels. This identification was supported by in vitro studies demonstrating that HipB binds to four operator sites, with the conserved inverted repeat sequence TATCCN$_8$GGATA, protecting a region of approximately 100 bp from DNase I digestion. Since this protected region includes the -35 and -10 promoter elements as well as the *hip* transcriptional start site, it is likely that HipB represses transcription from the *hip* promoter as do classical prokaryotic repressors, by steric occlusion of RNA polymerase.

Many prokaryotic DNA-binding proteins containing the helix-turn-helix structural motif recognize sequences with partial twofold symmetries (27). Often these proteins bind to operator DNA as a dimer or tetramer in two adjacent helical turns of the major groove along one face of the DNA helix. HipB appears to bind in a similar manner. Cross-linking and gel filtration studies showed that HipB forms dimers in solution. Thus, it is anticipated that HipB binds to each operator site as an oligomer of at least two subunits. However, definitive proof that a dimer or other oligomer is the active binding species requires further investigation.

HipB primarily modified the methylation of guanine residues (Fig. 4); 23 of the 27 affected guanine residues were protected. The 10 adenine residues that were affected by HipB were hypermethylated; none was protected. The spacing of protected guanine residues on opposite strands within each

5'-GCGGCTTAATATAGCCGACTTCAAACGCGCATCAACGCCTTATTTAGATCCTCCTTTGTTACCGATCGCGGATATCGCAGCGTTTATCCCGTAGAGCGGATAAGATGTG
O4 →   O3 →

3'-CGCCGAATTATATCGGCTGAAGTTTGCGCGTAGTTGCGGAATAAATCTAGGAGGAAACAATGGCTAGCGCCTATAGCGTCGCAAATAGGGCATCTCGCCTATTCTACAC
-140   -120   -80   -60

-35   -10
V TTTCCAGATTGACTTATCCTCACTAAAGGATAAAACTTATAATATCCCCTTAAGCGGATAAACTTGCTGTGGACGTATGACATGATGAGCTTTCAGAAGATCT-'3
O2 →   O1 →

AAAGGTCTAACTGAATAGGAGTGATTTCCTATTTTGAATATTATAGGGGAATTCGCCTATTTGAACGACACCTGCATACTGTACTACTCGAAAGTCTTCTAGA-'5
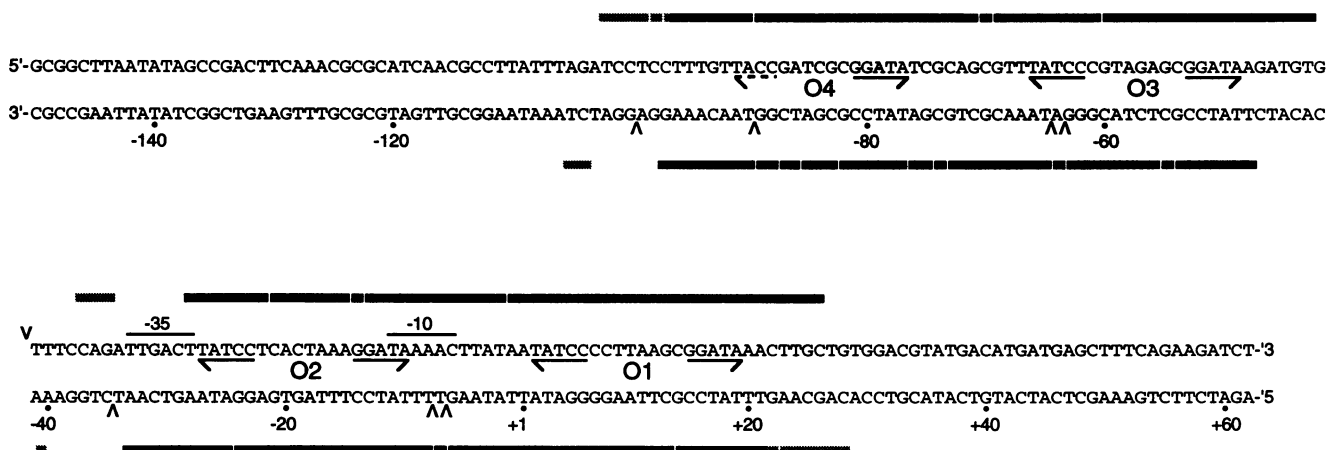-40   -20   +1   +20   +40   +60

FIG. 8. Comparison of the HipA-HipB and HipB DNase I protection patterns. The diagram summarizes the footprinting experiments performed with the HipA-HipB complex and compares these results with those observed with HipB. A portion of the *hip* regulatory region numbered with respect to the *hip* transcriptional start at +1 is shown. The regions overlined and underlined with a heavy bar represent sequences protected from DNase I digestion by HipA-HipB (grey and black bars) or HipB (black bars only). Phosphodiester bonds which have become hypersensitive to DNase I digestion in the presence of HipA-HipB are indicated (^). The *hip* promoter and operator regions are marked as described in the legend to Fig. 4.

operator approximates a single turn of the DNA helix. Therefore, since dimethyl sulfate specifically methylates the N-7 position of guanine residues in the major groove and the N-3 position of adenine residues in the minor groove (31), the interaction of HipB within an individual operator appears to occur in adjacent major grooves. Examination of the overall spacing between operators and protected residues suggests that HipB binding occurs on the same face of the DNA helix. Operators O1 and O2 and operators O3 and O4 are separated by 10 bp, and operators O2 and O3 are separated by 21 bp. The protected residues are at the same relative positions within each operator.

The proximity of the four operators and their arrangement on the same face of the DNA helix would facilitate interaction between bound proteins. Indeed, there is evidence suggesting that HipB binds to the operators in a cooperative manner. On the basis of gel retardation and DNase I protection experiments, HipB appears to bind to all four operator sites almost simultaneously. In gel retardation assays, only a single, retarded complex was observed. Furthermore, DNase I protection studies demonstrated that all four operator sites were protected at similar protein concentrations. The absence of intermediate complexes with one, two, or three operator sites filled suggests that HipB binds to the operators cooperatively. Cooperativity is supported by gel retardation studies showing that there is a steep, single-step transition from unbound to bound DNA. An increase of only about fourfold in HipB protein concentration is required to increase the bound fraction of DNA from 10 to 90% (Fig. 2B). Additional data supporting the notion that HipB binds cooperatively is the observation that the affinity of HipB for a fragment containing O1, O2, and O3, the *Eco*RV-*Bgl*II (bp −77 to +61 of *hip*) fragment, is 10-fold less than for a fragment containing all four operator sites, the *Bam*HI-*Bgl*II (bp −291 to +61 of *hip*) fragment.

Insertional inactivation of *hipA* at the *hip* locus increased expression from the *hip-lac* operon fusion at the λ *att* site almost twofold, suggesting that HipA also has a role in the regulation of the *hip* promoter. HipA does not appear to bind the *hip* regulatory region directly but indirectly via HipB as a

component of a HipA-HipB complex. The affinities of HipB and HipA-HipB for the *hip* regulatory region appear to be similar. However, there is evidence suggesting that HipA-HipB interacts with this region in a manner different than that of HipB (Fig. 8).

Regulation of the *hip* operon has additional complexities. Sequence analysis of the *hip* regulatory region revealed a putative IHF binding site (Fig. 1). Gel retardation and DNase I protection experiments confirmed that IHF binds to this site (unpublished data); however, in vivo studies have yet to demonstrate the physiological significance of this site. In addition to regulation by the *hip* gene products and possible regulation by IHF, the *hip* promoter is subject to inverse growth rate and, independently, inverse temperature control (unpublished data). This effect is independent of the *hip* gene products, and the basis for such control is not known.

There are few clues as to what physiological role *hip* might play in *E. coli*. There is evidence that production of HipA in excess of HipB is deleterious to the cell. The nature of this toxicity is not known. Elucidating the mechanism of HipA toxicity might be valuable in determining the physiological role of *hip*. One avenue of pursuit is discovery of genes or gene products with which HipA and/or HipB interact. Mutations at loci other than *hip* (33.8 min) result in high-frequency persistence. Wolfson et al. report the finding of a mutant allele of *hipQ* which displays the Hip phenotype (36). *hipQ* maps to min 2 on the *E. coli* chromosome. In addition, a mutation mapping to 76 min on the *E. coli* chromosome results in a 1,000- to 10,000-fold decrease in killing when DNA or peptidoglycan synthesis is inhibited (unpublished data). Further characterization of these loci leading to the identification of these genes and examination of these loci with respect to their interaction with *hip* is anticipated to be fruitful.

There are several reasons for believing that *hip* might affect cell division. The first is the nonlethal, cold-sensitive block in cell division caused by mutant alleles of *hipA* (30). The second is the fact that a single mutation affects the frequency of persistence to inhibition of both peptidoglycan synthesis and DNA synthesis (30). The third is the temporal linkage between completion of a round of DNA synthesis and several aspects of

peptidoglycan synthesis including susceptibility to the lethal consequences of inhibition of such synthesis (7, 18). In addition, there is evidence that unbalanced or excessive expression of *hipA* is toxic. This evidence and the relationship of *hip* to the division process suggest that *hip* gene products interact with one or more cell division genes or their products. In fact, it is of interest to note that the newly identified Hip loci, mapping at 2 and 76 min on the *E. coli* chromosome, are in regions known to contain cell division genes (2).

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